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## INTRODUCTION

Multicellular organisms depend on cell-cell communication to coordinate growth and differentiation. The developmental fate of many cells is determined in part by the action of location-specific intercellular signals. That is, cells decide what to become based, in part, on where they are located. The *wingless/Wnt1*, *hedgehog*, *decapentaplegic*/TGF and FGF pathways are responsible for providing much of this position-specific information. Our work focuses on the *wingless/Wnt1* pathway. *wingless* and *Wnt1* are members of the WNT gene family (reviewed in Moon et al. 1997; Nusse and Varmus 1992). Despite that fact that the WNT proteins were first identified over 15 years ago surprisingly little is known about these ligands, their receptors, how the signal is transduced across the membrane, or even how these large proteins (with apparent molecular weights from 30 to 60 kDa) are able to move about in the extracellular environment.

**The many roles of *wingless* in *Drosophila* development.** *wingless* (*wg*) was the first WNT gene identified in *Drosophila* and is the best characterized member of the WNT family. *Wg* functions in many different tissues and in many seemingly disparate developmental events. It is required for patterning of the embryonic epidermis, midgut invagination, neuroblast differentiation, and even Malpighian tubule growth. In some tissues *Wg* acts as a short range signal (van den Heuvel et al. 1988). In others it functions over a range of 10-20 cell diameters (Zecca et al. 1996). It has been hypothesized that in each case secreted *Wg* protein (*Wg*) acts as a position specific signal, establishing spatial coordinates.

**The WNT gene family.** More than 20 WNT related genes have now been identified in a variety of organisms. The WNT proteins share 22 conserved Cys residues and an overall amino acid sequence similarity of 50-60% (Sidow 1992). Ectopic expression experiments have shown that many of the WNT genes are proto-oncogenes. *Wnt1*, the mouse ortholog of *wingless*, is expressed in two regions: the fetal brain and in adult testes (McMahon and Bradley 1990). Null alleles of *Wnt1*, created by gene targeting in mouse embryonic stem cells, result in the loss of the midbrain and parts of the cerebellum (Thomas and Capecchi 1990; McMahon et al. 1990; McMahon et al. 1992). Other *Wnt* genes are expressed in a variety of embryonic structures, and in some adult tissues.

**Wg/Wnt1 signaling: the downstream response pathway.** Other genes in the Wg/Wnt1 pathway have also been identified (Reviewed in Peiffer 1995; Kinzler and Vogelstein 1996). Just as *wg* and *Wnt-1* are closely related, these genes have also been highly conserved through evolution. A working model of the downstream pathway is as follows. Extracellular Wg/Wnt1 ligand is thought to bind to one of a family of Frizzled (Fz) receptors. The receptor in turn activates Dishevelled (Dsh), which represses the activity of the Ser/Thr kinase Zeste White (3) (Zw(3)). When Zw(3) is repressed, Armadillo (Arm), becomes activated. Arm binds to HMG transcription factors such as LEF.

### **Wg/Wnt1 Signaling: The Extracellular Steps.**

**Extracellular proteoglycans participate in Wg/WNT signaling.** Work in our lab has shown that glycosaminoglycans can stimulate Wg signaling *in vitro* (Reichsman et al. 1996; Cumberland and Reichsman 1997). This work will be described in the Preliminary Results Section. Recent genetic studies in *Drosophila* argue that these interactions also occur *in vivo* (reviewed in Cumberland and Reichsman 1997). Three laboratories (Binari et al. 1997; Hacker et al. 1997; and Haerry et al. 1997) have now demonstrated that mutations in the gene encoding UDP-glucose dehydrogenase (UDP-GlcDH) disrupt both the synthesis of glycosaminoglycans and Wg/WNT signaling. Animals lacking maternal and zygotic UDP-GlcDH activity die in late embryogenesis, and the

mutant embryos have segment polarity defects like those of *Wg*<sup>-</sup> embryos. This enzyme catalyzes the conversion of UDP-glucose to UDP-glucuronic acid, an essential substrate for the biosynthesis of all glycosaminoglycans except keratin sulfate. Thus animals lacking UDP-GlcDH activity are unable to synthesize the glycosaminoglycan chains needed to form proteoglycans.

Given the high degree of conservation between the *wg* signaling pathway in *Drosophila* and the *Wnt1* pathway in vertebrates, we speculate that GAGs also function in *Wnt1* signaling. This idea is supported by the finding that several other WNTs also bind to heparin (Bradley and Brown 1990; Burrus and McMahon 1995). Moreover, proteoglycans are required for maintenance of WNT11 expression in the ureter tips (Kispert et al. 1996).

***frizzled* proteins are candidate Wnt Receptors.** Members of the *frizzled* (*fz*) gene family encode seven-pass transmembrane proteins with large cysteine-rich extracellular domains (Wang et al. 1996). The Fz proteins are excellent candidates for Wg/WNT receptors (Bhanot et al. 1996). Genetic studies have shown that *dsh*, a component of the *Wg* pathway, is downstream of *Dfz1* (Krasnow et al. 1995). This suggests that *Dfz1* might be in the *wg* pathway, or that the *wg* and *Dfz1* paths might intersect. Since *Dfz1* is not required for epidermal segmentation and therefore cannot be the only Wg receptor, it has been suggested that DFz1 and DFz2 have some redundant functions and that one or both can function as a Wg receptor. Mutations in *Dfz2* have yet to be isolated.; however, Bhanot et al. (1996) have shown that Wg can bind to transgenic S2 cells expressing either *Dfz1* or *Dfz2*. Furthermore, S2 cells expressing either *Dfz1* or *Dfz2* are responsive to Wg, while parental S2 cells are not (Bhanot et al. 1996; Nusse personnel communication; Chen and Cumberledge, unpublished). The strongest genetic evidence that *fz* genes might encode WNT receptors comes from work in *C. elegans*. Sawa et al. (1996) have shown that mutations in the *frizzled*-like gene *lin-17* have a phenotype which is complementary to *lin-44* mutants (*lin-44* is the *C. elegans* homologue of *Wg*). Genetic interactions have also been described between Mom-2, another Wnt-like gene and the *frizzled*-like gene Mom-5. These experiments support, but do not prove, the idea that the *fz* genes encode Wg receptors. Still missing is either genetic data that the *fz* genes are required for *Wg* signaling, or biochemical data measuring direct high affinity Wg-Fz binding.

**The secreted Frzb proteins inhibitors of Wg/WNT activity.** Recent studies in mouse, humans, and *Xenopus* have led to the identification of a new component in WNT signaling: the Frzb proteins. Frzb proteins are a family of secreted proteins which share ~50% amino acid homology with the extracellular cysteine-rich domain (CRD) of the Frizzled proteins (Rattner et al. 1997). *Frzb-1* is expressed in many mammalian tissues (reviewed in Moon et al. 1997). Ectopic XFzb-1 expression can induce dorsalization of the embryo (Leyns et al. 1997; Wang et al. 1997a) and antagonize WNT activity. In vitro experiments demonstrating that XFzb co-immunoprecipitates with Wnt proteins (Wang et al. 1997a) have led to the hypothesis that XFzb interacts directly with extracellular Wnt protein. This model is also consistent with in vivo work showing that *XFrzb-1* inhibition of *XWnt-8* is non-cell autonomous.

To what extent do Frzb proteins regulate Wg/WNT signaling in vivo? *Frzb-1*, can antagonize Wnt1 and Wnt8 function, but does not block signaling by Wnts -3A, -5A, or -11 (Wang et al. 1997b). Four mammalian *Frzb* genes have been identified already (Rattner et al. 1997); each is expressed in a tissue- and temporal- specific manner. Given their broad expression patterns the Frzb antagonists may be a vital part of many Wnt signaling pathways. It is not known whether *Frzb* homologs are present in *Drosophila*. Nonetheless, expression of GPI-linked forms of sFRP-2 or sFRP-3 in transgenic tissue culture cells is sufficient to confer cell-surface binding by Wg (Rattner et al. 1997).

## BODY - RECENT PROGRESS

### Overview

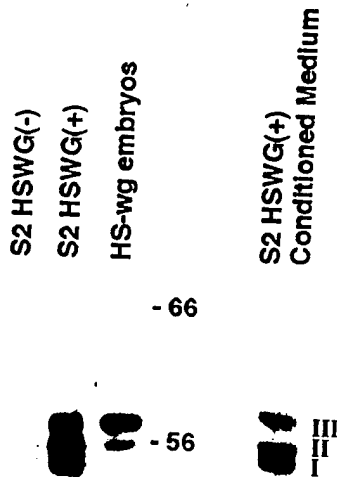
Our long term goals are to understand how the Wg signal is transmitted from cell to cell and how information is transduced in the receiving cells. Our work has focused primarily on purification of Wg, ligand-receptor interactions, and the role of extracellular cofactors in signal transduction. In addition, we have made significant progress towards understanding the pathway leading to the post-translational modification and secretion of Wg. We have also initiated preliminary studies on Wg-receptor interactions. I will summarize our relevant published work and discuss some of the studies now in progress.

### I. Post-Translational Modification and Secretion of Wg

Several years ago we generated  $\alpha$ Wg antibodies (Reichsman et al. 1996) and a tissue culture cell line that secretes soluble, active Wg protein (Cumberledge and Krasnow 1993; Reichsman et al. 1996). These reagents have made it possible for us to follow the expression, modification, and secretion of Wg both in vitro and in vivo.

#### Wg is a glycoprotein

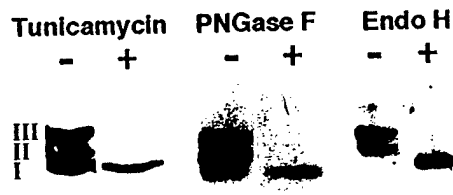
Three distinct electrophoretic forms of intracellular Wg can be detected as shown by Western analysis of Wg expression in embryos and in S2HSWg cells. The three forms, I, II, and III have apparent molecular weights of 52, 55, and 57 kDa respectively. Form III is the predominant form found in vivo.



**Figure 1.** Western blot of Wg isoforms expressed both in vitro and in vivo. S2 cells, S2HSWg cells, and *hs-Wg/TM3Sb* embryos (2-11 hours after egg laying), were heat-shocked 1 hour at 37°C and allowed to recover at 25°C. Whole cell extracts and Wg-conditioned medium were then prepared as described, subjected to 10% SDS-PAGE, transferred to nitrocellulose, and probed with rabbit  $\alpha$ Wg antibody.

Form I is the core protein and Forms II and III contain Asn-linked Mannose glycans. When S2HSWg cells are grown in the presence of tunicamycin, an inhibitor of Asn-linked glycosylation, only Form I is expressed (Figure 2A). In addition, when the three isoforms are cleaved with Peptide: N-glycosidase F (PNGase F), which removes all oligosaccharides, only the core protein remains (Figure 2B). The glycans appear to belong to the high Mannose class. Forms II and III are sensitive to Endo-*b-N*-acetylglucosaminidase H (Endo H), although a small amount of Endo H resistant 55 kDa protein is sometimes detected (Figure 2C). Endo H removes high Mannose but not complex glycans. All three isoforms are synthesized in the ER. When cells are treated with DTT, which inhibits protein folding and blocks ER to Golgi transport, the three forms are still made (not shown). This finding also indicates that the sugars are high Mannose rather

than complex since the Man9 block is added in the ER, while processing typically occurs in the Golgi.



**Figure 2** Characterization of the sugar moieties found on Wg.

(A) Tunicamycin blocks post-translational modification of Wg. S2HSWg cells were incubated for 2 hours in the presence or absence of tunicamycin, heat-shocked one hour and allowed to recover for one hour. Whole cell extracts were prepared, fractionated by 10% SDS-PAGE and transferred to nitrocellulose; blots were probed with  $\alpha$ Wg antibody.

(B) PNGase F cleaves glycoforms forms II and III. Wg was immunoprecipitated from S2HSWg whole cell extracts, then treated with and without Peptide:N-glycosidase F (PNGase F). After digestion, samples were analyzed by Western blotting as in A.

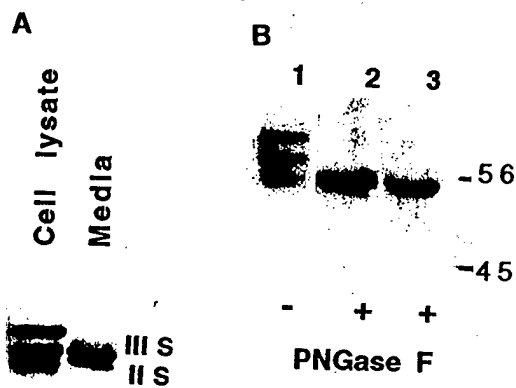
(C) Glycoforms II and III are Endo H sensitive. Wg was immunoprecipitated from S2HSWg cells, then treated with and without Endo-*b-N*-acetylglucosaminidase H (Endo H). After digestion, samples were analyzed by Western blotting as in A.

Our current working model is that Form II has one oligomannose glycan and Form III has two glycans. There are three potential glycosylation sites in Wg. We are using site directed mutagenesis to identify which sites are utilized. We have already identified two glycosylation sites: one at Asn 113 and one at Asn 414, and we are in the processes of constructing the third mutant.

#### **Evidence for proteolytic processing of secreted Wg.**

Two Wg glycoforms and the core protein are also present in conditioned medium harvested from S2HSWg cells (Figure 3A). Note however that the molecular weights of the two extracellular glycoforms (Forms IIS and IIIS) are 53 and 55 kDa, in contrast to the 55 and 57 kDa Wg bands found in whole cell extracts. Form IIIS (55 kDa) is the most abundant. The decrease in molecular weight is not due to additional processing of the sugars. We have found that the secreted polypeptide is smaller than the core protein isolated from whole cell lysates. Figure 3B shows that PNGase F treatment of IIS and IIIS produces a single 51 kDa core protein. Thus, the apparent molecular weight of the secreted polypeptide core is about 1 kDa smaller than the peptide found in whole cell lysates.





**Figure 3** Analysis of the Secreted Forms of Wg.

(A) Western blot showing Wg isoforms present in S2HSWg conditioned medium and whole cell extracts.

Samples were fractionated on 10% SDS-PAGE, and then transferred to nitrocellulose; the blot was probed with  $\alpha$ Wg antibody

(B) PNGase F cleavage of Forms II S and III S releases the core protein. Wg was immunoprecipitated from cell lysates (lane 1,2) and conditioned medium (lane 3).

Samples were incubated in the presence and absence of PNGase F. After cleavage, the samples were analyzed by Western blotting as in A.

Recent work in our lab suggests that cleavage is not requisite for secretion. In the course of carrying out structure/function studies on several Wg mutants (see below) we have characterized several Wg mutant alleles which encode proteins with single amino acid substitutions near the C-terminus. These mutant proteins are expressed and secreted, but are inactive. We were intrigued to find that the secreted mutant proteins are not cleaved (data not shown).

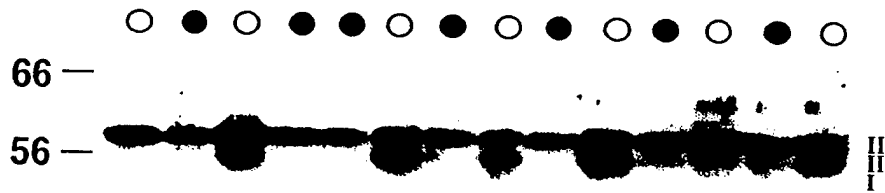
What is the functional significance of the proteolytic processing? Is cleavage required for activity? In order to address these questions, it is necessary to first characterize the cleavage in more detail. It is likely that the cleavage event occurs near the N or C-terminus of the protein. We are testing this hypothesis now. We are examining the expression and secretion of several affinity tagged forms of Wg. We have constructed transgenic S2 cells which express Wg with a Carboxy-terminal His tag. These cells express and secrete Wg-His. The secreted Wg-His polypeptide is smaller than the cellular form; however it still retains the C-terminal tag. Thus, it seems likely that the cleavage event occurs near the N-terminus. We are constructing several Amino-tagged forms of Wg. The His-tag will be inserted at several sites near the putative cleavage site for the signal sequence. The various His-Wg constructs will be expressed in S2 cells and we will characterize the cytosolic and secreted forms of each tagged protein.

We are also characterizing Wg processing in vivo. Wg is expressed at very low levels in vivo, and we cannot detect endogenous Wg expression by conventional Western analysis. We have recently developed protocols for immunoprecipitating Wg from whole embryo lysates. We will use this protocol to immunoprecipitate Wg from wild-type animals. The immunoprecipitated Wg will be treated with PNGase F.

#### **A 55 kDa Wg glycoform accumulates in *porcupine*<sup>-</sup> animals**

Genetic studies have shown that *porcupine* functions upstream of *wg* in the signaling pathway (Perrimon et al. 1989; Eberl et al. 1992; Noordermeer et al. 1994; Siegfried et al. 1994). *porcupine* encodes a multipass transmembrane protein located in the secretory machinery (Kadowaki et al. 1996). In *porcupine*<sup>-</sup> animals Wg signaling is blocked and extracellular Wg cannot be detected by whole mount immunocytochemistry

(van den Heuvel et al. 1993; Siegfried et al. 1994). These observations have led to the hypothesis that *porcupine* is required for Wg secretion in vivo. A vertebrate homolog of *porcupine* has also been identified recently. Given that so much of the Wg/WNT signaling pathway has been highly conserved through evolution, it is likely that the vertebrate *porcupine* participates in Wnt secretion.



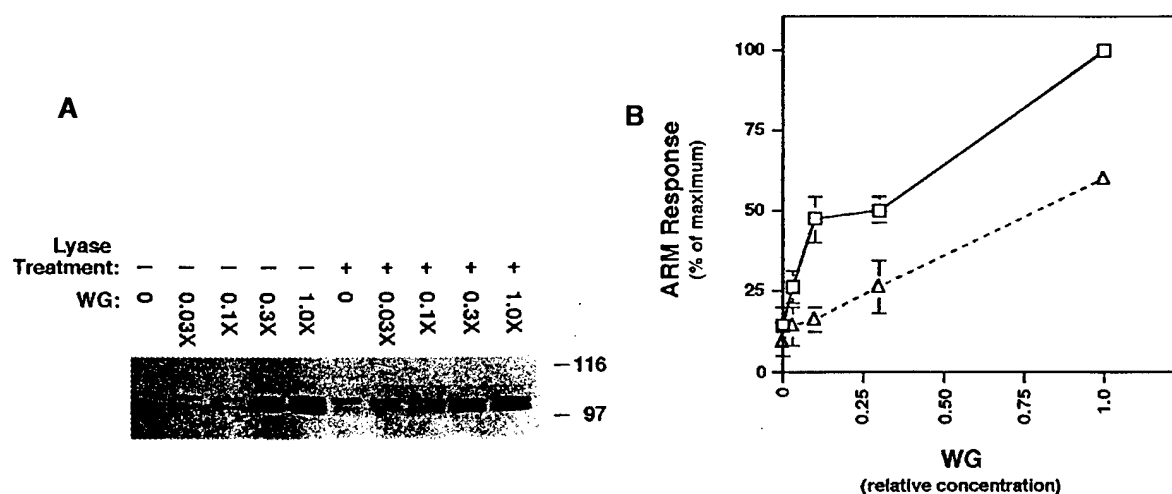
**Figure 4.** Post-translational modification of Wg is affected by loss of *porcupine*. Western blot of whole larval extracts prepared from individual wandering third instar larva. Open circles: *HS-Wg*; *porc<sup>PB16</sup>* and *HS-Wg*; *FM7* males from the cross *HS-Wg/TM6Tb*; *+1Y* x *+1+*; *porc<sup>PB16</sup>/FM7*; closed circles: control *HS-Wg* / *TM6Tb* males.

We hypothesize that Forms II and III are synthesized in the ER, transported to the Golgi, processed into Forms IIS and IIIS and then secreted. In *porcupine*<sup>-</sup> animals Wg will continue to be translated, glycosylated and processed. Wg secretion is blocked in these animals, and we speculate that the fully processed isoforms are forced to accumulate within the cell. In this model *porcupine* does not regulate Wg glycosylation; but instead functions at a later step in the secretory path. For example *porcupine* might target Wg to the correct secretory vesicles or perhaps facilitate vesicle transport to the plasma membrane. This model makes several predictions about Wg processing which we are now testing.

## II. Purification and Characterization of Wg

### Measuring Wg activity in vitro.

One of the goals in the lab has been to purify and characterize Wg protein. In order to purify Wg, we needed a reliable activity assay. We have measured Wg activity using the Arm assay described by van Leeuwen et al. (1994). Previous genetic studies have shown that *zw(3)*, a Ser/Thr kinase, promotes the phosphorylation and inhibition of Arm protein. Wg inhibits *zw(3)* activity thereby activating Arm. When clone-8 cells, a *Drosophila* cell line derived from imaginal discs, are incubated with soluble Wg in conditioned medium from S2HSWg cells, there is a large increase in the ratio of dephosphorylated to phosphorylated Arm and a concomitant increase in the total amount of cellular Arm protein. We have quantitated Wg activity in this assay by measuring the increase in the dephosphorylated (faster migrating) form of Arm. Figure 5 shows a concentration dependence curve for Wg protein present in the conditioned medium of S2HSWg cells. Using the Arm assay, we can detect Wg activity at concentrations in the pM range (Figure 5).



**Figure 5** Dose-response curve for Wg activity S2HSWg cells were assayed for Wg activity. 1X medium = approximately 4nM Wg, as measured by immunoprecipitation of [<sup>35</sup>S]-Met-labeled Wg (see Reichsman et al. 1996).

A. Lysates of the clone-8 cells were subjected to 8% SDS-PAGE (70 µg protein/lane) and Western analysis. The blots were probed with N27A1 αArm and αHsp70 monoclonal antibodies, followed by goat αmouse-HRP antibodies. Immunoreactive proteins were visualized using enhanced chemiluminescence.

B. Data from three similar experiments are represented graphically. The increase in the amount of dephosphorylated (faster migrating) Arm was used to quantitate Wg activity. Densitometry was performed using NIH Image. No Arm response was observed in clone-8 cells exposed to conditioned medium from S2HSWg(-) control cells, not shown).

## Purification Scheme

We have made substantial progress in purifying Wg from S2HSWg cells using classical biochemical techniques. The Arm assay has been used to monitor Wg activity, and an αWg Ab has been used to follow Wg protein. At the time our original RO1 proposal was submitted, we were approximately half-way through the purification. Along the way we had developed techniques for harvesting, concentrating and storing Wg. Using heparin-agarose affinity chromatography, we had achieved a 200 fold purification of Wg and could routinely obtain 1-5 µg quantities of Wg protein (approximately 7% pure). To our knowledge, this is the first time anyone has successfully fractionated Wg and retained biologic activity.

While carrying out these studies, we also designed and tested several affinity tagged forms of Wg. The first two constructs we made, one with a KT3 tag and one with six HIS residues at the carboxyl terminus, were not useful. Both were expressed, but unfortunately neither tagged protein was secreted. Recently Bradley and Brown (1995) were able to construct an HA tagged Wnt1 protein which is active in the RAC paracrine transformation assay. Although the HA tag itself is not useful for purification, we designed a new His-tagged Wg construct using the HA-Wnt as a model. A short oligonucleotide encoding a Proline followed by a three amino acid spacer arm and six HIS residues, was inserted at the equivalent location in Wg. The expression plasmid was used to transfect S2 cells and stable transgenic cell lines were isolated. These S2HSWgHis cells secrete soluble, His-tagged Wg. Furthermore, conditioned medium containing the His-tagged Wg is active in our Arm activity assay (Figure 6). This is a significant breakthrough for the

purification. The presence of the His-tag will allow us to incorporate a Ni-affinity chromatography step.

**Figure 6** Secreted Wg<sup>His</sup> can induce an Arm response in S2DFz2 cells. Western blot of S2DFz2 cells after a 2 hour incubation with conditioned medium from control cells, S2HSWg cells or S2HSWg<sup>His</sup> cells. Blots were probed with  $\alpha$ Arm Ab and  $\alpha$ Hsp70 Ab (loading control)

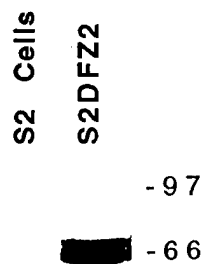
**Multiple Frizzled receptors can interact with Wg in vitro.**

Some of the outstanding questions about Wg/WNT signaling have to do with ligand-receptor specificity. Does each Wg/WNT protein recognize only one Frizzled (Fz) protein or can one ligand activate multiple receptors? In order to address these issues we have constructed transgenic S2 cell lines which express either DFz2 or DFz1. A DNA fragment containing the full length *Dfz1* cDNA was cloned into the expression vector pMK33, such that expression of DFz1 is under control of the metallothionine promoter. A similar pMK33-DFz2 plasmid was obtained from Roel Nusse. Stable transgenic S2 pMK33-DFz2 and S2 pMK33-DFz1 cells were constructed (S2DFz2 and S2DFz1 cells respectively).

Expression of Fz1 protein in the S2DFz1 cells was monitored by probing Western blots of whole cell extracts with  $\alpha$ DFz1 Ab (kindly provided by P. Adler). Two bands were detected; the apparent MWs of these bands corresponds to the DFz1 bands reported previously (Krasnow et al. 1994).

Since antibodies against DFz2 were not available, we raised  $\alpha$ DFz2 polyclonal antibodies in rabbits. Briefly, a 30 kDa His-tagged peptide corresponding to the extracellular domain of DFz2 was expressed in *E. coli* and then purified. This peptide was injected into rabbits using a standard protocol for antibody production. After several round of antigen injections, the serum was tested for cross-reactivity with DFz2. *Drosophila* embryos were fixed and stained with the  $\alpha$ DFz2 antibody (diluted 1:10,000). Immunocytochemical staining of the embryos gave a staining pattern similar to the reported expression pattern for *Dfz2* (Bhanot et al. 1996).

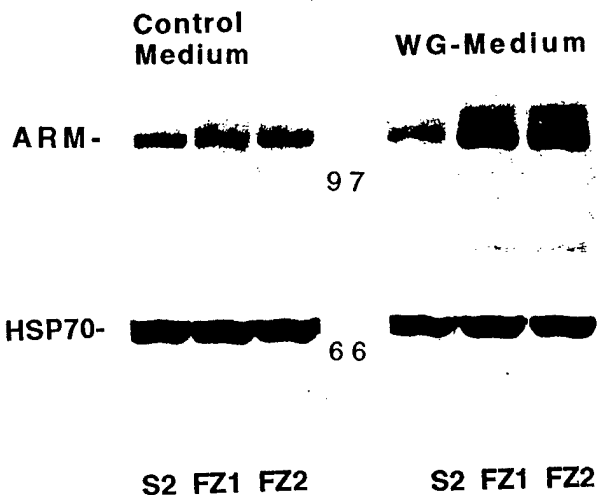
Subsequently, Western blots containing whole cell extracts from S2DFz2, S2DFz1, and S2 cells were probed with  $\alpha$ DFz2 Ab (diluted 1:50,000). A cross-reacting doublet was detected in the S2DFz2 cells (Figure 7). The mobility of the upper band corresponds to the predicted molecular weight for DFz2 (70 kDa). No band was detected in control S2 or S2DFz1 cells (Figure 7; data not shown).



**Figure 7.**  $\alpha$ DFz2 antibody recognizes DFz2 protein.

Whole cell extracts prepared from S2 and S2DFz2 cells were fractionated by 3-10% SDS-PAGE. The protein was transferred to PVDF membrane and the blot was probed with rabbit  $\alpha$ DFz2 antiserum. 20 mg of total protein was loaded per lane. Note that two DFz2 bands are detected. Studies by Adler and co-workers have shown that DFz1 is also expressed as a doublet, both in S2DFz1 cells and in vivo (Krasnow et al. 1994).

With the three cell lines in hand, we have assayed each for responsiveness to Wg. Figure 8 shows that cells expressing either DFz2 or DFz1 display an Arm response to Wg signaling, while control S2 cells do not. Thus in the in vitro Arm assay, DFz1 can substitute for DFz2.



**Figure 8.** S2 cells expressing either DFz1 or DFz2 are responsive to Wg.

Cells were incubated with Wg-conditioned medium or control medium. After two hours, the cells were harvested and Arm expression was monitored by Western blotting. Whole cell extracts were fractionated on 8% SDS polyacrylamide gels, then transferred to PVDF membrane and probed with  $\alpha$ Arm Ab and  $\alpha$ Hsp70 Ab (loading control).

### III. Interactions with Extracellular Cofactors - The Role of Glycosaminoglycans (GAGs)

As an off-shoot of our purification studies, we have discovered that Wg can interact with extracellular glycosaminoglycans (Reichsman et al. 1996). These glycosaminoglycans stimulate Wg signaling and may even be required for Wg activity. This is intriguing, since a variety of growth factors are known to bind to proteoglycans (Kjéllan and Lindahl 1991). Proteoglycans contain covalently linked glycosaminoglycan chains, such as heparan sulfate and chondroitin sulfate making them highly negatively charged. Some extracellular proteoglycans function as co-receptors for growth factors (Klagsbrun and

Baird 1991; Schlessinger et al. 1995). For example, secreted TGF $\beta$  can form a tripartite complex with the cell surface proteoglycan betaglycan-1 and the type II TGF receptor (López-Casillas et al. 1993). In some types of cells both betaglycan and the type II receptor are required for transmembrane signaling (Yayon et al. 1991). Similarly, the proteoglycan perlecan binds to FGF $\beta$  and promotes FGF $\beta$  binding to its high affinity receptor (Yayon et al. 1991).

Four independent lines of evidence have led us to propose that glycosaminoglycans act as accessory factors for Wg. Three of these findings are now published (Reichsman et al. 1996). I will summarize them briefly. (1) When clone-8 cells are pre-treated with glycosaminoglycan (GAG) lyases to enzymatically remove SO<sub>4</sub>-GAGs, the cells become refractory to Wg signaling. (2) Treating clone-8 cells with sodium perchlorate, which blocks sulfation of proteoglycans also inhibits Wg signaling. (3) Wg binds tightly to heparin agarose. A fourth line of evidence comes from our work on purifying Wg. We have estimated the activity for Wg at each step in the purification (where activity equals the amount of Wg protein needed to obtain a half-maximal Arm response). As Wg is purified away from other components, it shows only weak activity in the clone-8 cell assay; however activity can be restored by the addition of exogenous heparin. The presence of heparin stimulates Wg activity as much as 5-fold. Are GAGs required for activity? It is possible that the partially purified Wg still retains some activity because there are still GAGs present in the preparation. As we complete the purification, we will determine whether "purified" Wg retains some activity, or if there is an absolute requirement for GAGs.

The GAG effects are quite specific: heparin and heparan sulfate stimulate Wg activity, while chondroitin sulfate does not. What factors might account for this specificity? All are highly negatively charged, being composed of irregularly repeating disaccharide units that are N-acetylated, and N- and O-sulfated (Silbert et al. 1995). It is unlikely that the degree of sulfation is responsible for the specificity of the interactions; however, the sugar makeup of the three GAGs correlates well with respect to their actions on Wg. All three contain N-acetyl-glucosamine, but only heparin and heparan sulfate contain N-acetyl-iduronic acid whereas chondroitin sulfate contains N-acetyl-galactosamine. This suggests that the hexosamine composition of the GAGs maybe an important binding determinant for specificity. Note that the  $\alpha$ 1-4 linkage in heparan sulfate will confer a much different chain shape than the  $\beta$ 1-4 linkage found in chondroitin sulfate.

The work we have carried out has all been performed in vitro. Additional evidence that glucosaminoglycans (e.g. heparan sulfate) but not galactosaminoglycans (e.g. chondroitin sulfate) participate in Wg signaling comes from genetic studies by X. Lin and N. Perrimon. They have identified a gene, *sulfateless*, that also affects Wg signaling (X. Lin and N. Perrimon, unpublished). *sulfateless* encodes a *Drosophila* homolog of the vertebrate N-deacetylase/N-sulfotransferases, a family of enzymes that catalyze the sulfation of heparin and heparan sulfate. These results argue that heparan sulfate biosynthesis and sulfation are necessary for normal Wg signaling in vivo.

#### **Glycosaminoglycans can influence receptor specificity**

S2DFz2 and S2DFz1 cells respond to glycosaminoglycans in different ways. When partially purified Wg is supplemented by heparin, there is an increase in signaling activity. S2DFz1 cells also respond to partially purified Wg. However, the S2DFz1 response is heparin independent. These results suggest that the activity of the glycosaminoglycans is receptor-type specific. Previous genetic studies by others has shown that Dfz1 is not required for Wg signaling in most cell types. Together, these

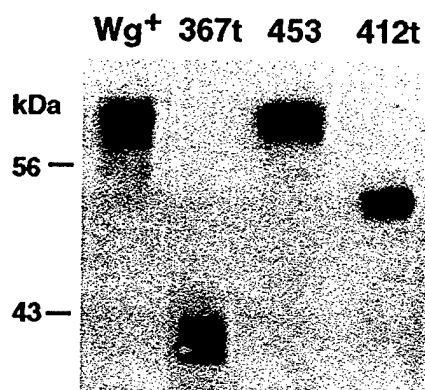
results lead us to hypothesize that the receptor specificity observed in vivo may be due in part to the contribution of cell surface proteoglycans.

#### IV Structure Function Studies-Using Mutants to Probe the Mechanism of Wg Signaling

We have characterized four loss of function *Wg* alleles (Moore et al, submitted). All four mutations map near the carboxyl (C-) terminus of the protein. The aberrant phenotypes resulting from these mutations suggest that sequences at the C-terminal end of Wg play an important role in Wg signaling. The amino acid sequence in this region is highly conserved between Wg and its vertebrate ortholog, Wnt-1, and includes twelve cysteine residues which are conserved among all WNT gene family members.

In order to understand why the mutants do not signal, we examined the biochemical properties of each protein. We began by asking whether the mutant proteins are stable and whether or not they are secreted.

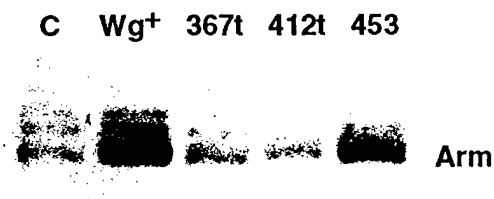
We expressed wild-type Wg and three of the mutant proteins (Wg<sup>453</sup>, Wg<sup>412t</sup>, and Wg<sup>367t</sup>) in *Drosophila* S2 cells and then assayed for secretion. Western analysis of conditioned medium harvested from each transgenic cell line confirmed that the mutant proteins were secreted (Figure 9). Note that the two truncated proteins, Wg<sup>412t</sup> and Wg<sup>367t</sup>, migrate with a lower apparent molecular weight than wild-type Wg. In general the amount of soluble mutant Wg present in the conditioned medium was similar to that of wild-type, suggesting that the C-terminal mutations did not significantly alter the stability of the protein (Moore, Xu, & Cumberledge, unpublished observations).



**Figure 9.** The C-terminal Wg mutants are secreted. Western blot of conditioned medium from stable transgenic S2 cell lines expressing either wild-type (Wg<sup>+</sup>) or mutant Wg. Blot was probed with  $\alpha$ Wg antibody. Conditioned medium from  $2 \times 10^6$  cells was loaded per lane. The position of molecular weight markers is indicated at left. The two truncation mutants Wg<sup>367t</sup> or Wg<sup>412t</sup>, migrate with a smaller apparent molecular weight than wild-type Wg.

### The C-terminal mutants lack most or all signaling activity

Each mutant was tested for activity using our paracrine assay for Arm stabilization. When S2DFz2 cells are incubated in the presence of wild-type Wg they display a marked increase in Arm levels (Bhanot et al. 1996) (Figure 10). As predicted from genetic studies, addition of either Wg<sup>412t</sup> or Wg<sup>367t</sup> does not affect the amount of Arm found in S2DFz2 cells. However, cells treated with Wg<sup>453</sup> do show an increase in total Arm (Figure 10). Wg<sup>453</sup> is less active than wild-type Wg in this assay; equivalent amounts of wild-type protein produce a stronger Arm response. This is consistent with our classification of Wg<sup>453</sup> as a hypomorphic allele.



**Figure 10.** The C-terminal mutants lack signaling activity. S2 cells expressing the Wg receptor, DFz2, were incubated with wild-type (Wg<sup>+</sup>) and mutant Wg conditioned medium, and analyzed by Western blot using  $\alpha$ Arm antibody, and  $\alpha$ Hsp70 antibody for loading control. Treatment with Wg<sup>+</sup> and Wg<sup>453</sup> conditioned medium resulted in an increase in abundance of Arm. Levels of Arm did not increase when the S2DFz2 cells were treated with Wg<sup>367t</sup> or Wg<sup>412t</sup>, or with control conditioned medium (C) from a Wg antisense construct. 70  $\mu$ g protein per lane.

Previous studies have shown that glycosaminoglycans are part of the Wg pathway (Jue et al. 1992; Reichsman et al. 1996; Binari et al. 1997; Hacker et al. 1997; Haerry et al. 1997). The mechanism(s) by which glycosaminoglycans stimulate Wg signaling is not known; however, workers have long speculated that interactions between secreted growth factors and extracellular glycosaminoglycans may determine the extracellular localization of the ligand. Because the extracellular distribution of Wg<sup>367t</sup>, Wg<sup>453</sup> and Wg<sup>445</sup> is abnormal, we tested each mutant for its ability to bind glycosaminoglycans. Conditioned medium was harvested from each cell line and the mutant Wg was fractionated by heparin agarose affinity chromatography. We found that mutations in the C-terminal end of Wg had little effect on heparin binding (data not shown). Like wild-type Wg, the mutant proteins bound quantitatively to heparin agarose, eluting only with high salt washes. We conclude that the C-terminal 101 amino acids of Wg are not needed for heparin binding. Furthermore, we find no correlation between the ability to bind heparin and the distribution of extracellular Wg.

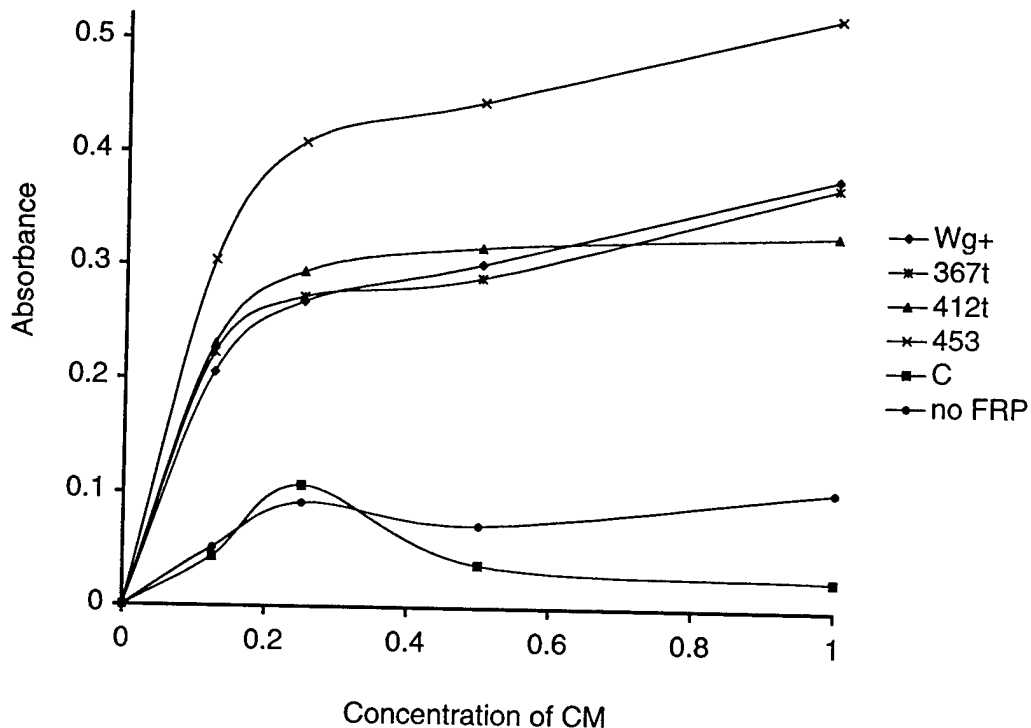
### Although inactive, Wg<sup>412t</sup> and Wg<sup>367t</sup> still bind a Frizzled Related Protein

Why do Wg<sup>412t</sup> and Wg<sup>367t</sup> lack activity? One possibility is that mutations in the C-terminal region of Wg disrupt the Frizzled binding domain. This hypothesis could be tested directly by assaying each mutant for DFz2 binding. Unfortunately purified DFz2 is not yet available. Instead, we have instead used FRP-1 purified from a MDCK cells (Finch et al.



1997). There are several reasons why FRP is a good choice for modeling Wg-Frizzled interactions. Like other Frizzled Related Proteins, FRP-1 contains the Frizzled cysteine rich domain (CRD), a putative ligand binding site. The amino acid sequences of the FRP-1 and DFz2 CRDs are 53% similar. Furthermore, purified FRP-1 is able to modulate both Wnt-1 (Finch et al. 1997) and Wg signaling (Rubin et al. in preparation).

We used the ELISA assay developed by Rubin and co-workers (in preparation) to measure Wg-FRP binding. Figure 10 shows that all three C-terminal mutants tested, Wg<sup>412t</sup>, Wg<sup>367t</sup>, and Wg<sup>453</sup>, retain their ability to bind to FRP. We have not measured the absolute K<sub>d</sub> values for binding. However, even in the case of Wg<sup>367t</sup>, where the last 101 amino acids have been deleted, the relative affinity of FRP binding is similar to that observed with wild-type Wg. Three conclusions can be drawn from these results. First, interaction with Frizzled Related Proteins does not require the C-terminal region of Wg. Second, the lack of activity in the mutants is not a consequence of their inability to recognize Frizzleds. Third, if a signal is to be transmitted, Wg must do more than simply bind Frizzled.



**Figure 11.** The mutant proteins recognize the secreted Frizzled-Related Protein sFRP-1.

ELISA assay of Wg binding to sFRP-1. Microtiter wells were coated with 300ng of purified sFRP-1 and wild-type or mutant Wg was then added to each well. After incubation, unbound Wg was removed and the amount of bound Wg was measured by ELISA (Rubin et al., submitted). No binding was observed with control conditioned medium isolated from S2 cells transfected with an antisense Wg cDNA. Each absorbance point plotted is an average of the absorbance readings from three wells treated identically. Data are representative of three independent assays. For all experiments, the relative concentrations of Wg in 1x conditioned medium were within two fold, as determined by dot blot analysis

## Conclusions

### **Wg undergoes multiple post-translational modifications.**

We have made significant progress in defining the steps required for maturation and secretion of Wg. We have shown that two glycoforms are synthesized and secreted in S2HSWg cells. Furthermore, the soluble Wg found in the medium has been cleaved. We have shown that peptide backbone of the secreted Wg is approximately 1 kDa smaller than the cytosolic protein core. We have characterized several mutant Wg proteins and shown that these lack signaling activity and are not cleaved. These results lead us to hypothesize that proteolytic processing may be necessary for activity. We are testing this idea now. Both glycoforms can also be detected in embryo extracts and we are currently investigating whether or not Wg is also cleaved in vivo.

### **Wg can signal through different Frizzled receptors.**

Although both S2DFz2 cells and S2DFz1 cells are responsive to Wg, there may be several orders of magnitude difference between the Kds for the two receptors. We are continuing with the purification and plan to carry out ligand-receptor binding studies in the near future. We will measure and compare the affinity of Wg binding to S2DFz2 cells and S2DFz1 cells. We will also address the question of whether extracellular cofactors can influence the specificity of ligand-receptor binding. For example, TGF $\beta$  recognizes both type I and type II TGF receptors. The proteoglycan betaglycan preferentially promotes TGF $\beta$  binding to the type II TGF receptor (López-Casillas et al. 1993).

### **Glycosaminoglycans stimulate Wg signaling.**

Our biochemical studies, in conjunction with recent genetic studies, have shown that glycosaminoglycans are an important component of the Wg/Wnt signaling pathway. The ability of the glycosaminoglycans to stimulate Wg signaling is dependent on the specific sugar sequences: heparan is much more effective than either chondroitin sulfate or sucrose octasulfate.

### **Glycosaminoglycans can be important determinants for defining receptor specificity.**

Two general models have been proposed to explain the role of proteoglycans play in growth factor signaling (see discussion in Reichsman and Cumberledge, 1997). In one model, binding to the proteoglycan serves to control or limit the distribution of the ligand. In the second model, the proteoglycan functions as a co-receptor. It is directly involved in receptor activation, perhaps through formation of a tripartite complex at the cell surface. The two proposed activities are not mutually exclusive; both might occur in vivo. We have found that heparin stimulates Wg signaling in S2DFz2 cells, but does not S2DFz1 cells. These results argue that proteoglycans can regulate the specificity of ligand-receptor interactions.

### **Probing the Mechanism of Wg Signaling: Evidence for a multi-step process.**

We have demonstrated that the C-terminal portion of the Wingless protein (Wg) is essential for signaling. Analysis of the heparin binding properties of four C-terminal suggests that the determinants for glycosaminoglycan binding are not affected by the C-terminal mutations. Furthermore, the C-terminal mutants bind to Frizzled-Related Protein-1 with similar affinity as wild-type Wg. These results suggest that Wg binding to Frizzled is not sufficient for signal transmission; additional steps are needed.

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